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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Post transcriptional control of gene expression is particularly important for oncoproteins and cell cycle proteins because their sustained synthesis favors cell growth rather than differentiation, a hallmark of the neoplastic phenotype. Control is exerted via the opposing actions of the RNA-binding proteins AUF1 and HuR. AUF1 triggers degradation of mRNA subsets while HuR promotes mRNA stabilization. Phase I of this work is to examine the effects of AUF1 and HuR expression levels on global gene expression in human breast carcinoma cells. Phase II is to assess roles of AUF1 and HuR in cellular proliferation and tumorigenesis in vivo. Previously, we discovered that AUF1 knockdown elevates expression of c-myc proto-oncogene by in vivo association with the mRNA, accelerates breast cancer cell proliferation, and alters their cell-cell adhesion properties. To explain the biological effects of AUF1 knockdown, we performed cDNA microarray analyses during the final funding period to identify AUF1's target mRNA subsets and their regulation by AUF1.

15. SUBJECT TERMS
Breast cancer; RNA-binding proteins; AUF1; HuR; mRNA stability

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INTRODUCTION

The level of a messenger RNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. The turnover rate of an mRNA can, in turn, determine its lifetime as a template for protein synthesis. It is particularly important to understand how the levels of mRNAs encoding oncoproteins and cell cycle proteins are regulated because sustained synthesis of these gene products favors proliferation rather than differentiation, a hallmark of the neoplastic phenotype. Many cell cycle and proto-oncogene mRNAs exhibit extremely short half-lives. Their decay is controlled in part by A+U-rich elements (AREs) located in the 3'-untranslated region. Moreover, the half-lives of their mRNAs are frequently subject to regulatory control. This control is exerted via a balance between the action of at least two ARE-binding proteins, AUF1 and HuR. AUF1 targets degradation of ARE-mRNAs such as the c-*myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of ARE-mRNAs. c-*myc* and cyclin D1 are of particular importance, since both play causative roles in mammary tumorigenesis. Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cellular proliferation, while HuR may act as a novel oncoprotein by stabilizing those mRNAs. To address this hypothesis, we proposed to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on proliferation and tumorigenesis in a nude mouse model. There were two proposed phases to this work: (I) to examine the effects of AUF1 and HuR expression levels on global gene expression in cultured human breast carcinoma cells; and (II) to assess the roles of AUF1 and HuR in proliferation and tumorigenesis *in vivo*. For Phase I we succeeded in both knockdown and overexpression of AUF1; stable knockdown of HuR did not prove possible. Interestingly, knockdown of AUF1 elevates translation of c-*myc* mRNA without effect upon mRNA abundance, to our surprise. For Phase II, we characterized clones for their growth phenotypes in preparation of examining their ability to induce tumors as xenographs on nude mice. Previously, we discovered that knockdown of AUF1 accelerates proliferation of breast carcinoma cells and ablates their cell-cell adhesion. To determine the molecular basis for the biological effects of AUF1 knockdown, we devoted the final, no-cost extension year to identification of the mRNAs to which AUF1 binds. The results indicate that AUF1 targets both signal transduction pathways and some structural proteins. The signaling pathways may contribute to the proliferation effects, while the structural proteins may contribute to the cell-cell adhesion effects.

BODY

The approved SOW is as follows:

Task 1. To examine the effects of AUF1 and HuR expression levels on gene expression in cultured breast carcinoma cells (Months 1-24):
    a. Construct Tet-Off plasmids for overexpression of AUF1 and HuR (Months 1-4).
    b. Construct plasmids for RNA interference (RNAi)-based knockdown of AUF1 and HuR (Months 1-4).
    c. Transfect plasmids into breast carcinoma Tet-Off cells and select individual clones (Months 4-24).
    d. Characterize clones for AUF1 and HuR expression levels to define those with low, medium, and high levels of AUF1/HuR expression (Months 6-24).
    e. Using RNA from engineered cell lines, perform DNA microarray analyses of genes affected by altered AUF1/HuR expression, in particular, those involved in the cell cycle, invasion, metastasis, and angiogenesis (Months 6-24).
    f. Identify those transcripts that are direct binding targets of AUF1 and HuR (Months 6-24).

Task 2. To assess the roles of AUF1/HuR in cell growth and tumorigenesis (Months 6-36):
    a. Characterize the growth phenotypes of engineered cell lines obtained from Task 1d (Months 6-24).
    b. Characterize the cell cycle distribution of these cell lines by flow cytometry (Months 6-24).
    c. Introduce clones onto nude mice and score tumor formation as a function of AUF1/HuR expression levels and growth/cell cycle phenotypes (Months 6-36).

As this is a final progress report (from a 4th, no-cost extension year), I will summarize work conducted over the entire research period but add details for the 4th year. For Tasks 1-a and -c, we completed preparation of expression constructs for all four AUF1 isoforms and HuR. We transfected these and performed western blot analyses of lysates to score for AUF1 or HuR overexpression (Task 1-d). Unfortunately, we were unable to overexpress HuR. However, we succeeded in overexpressing each of the AUF1 isoforms compared to the control (Con) transfection as shown in the western blot of Figure 1; α-tubulin served as a loading control.

**Figure 1**. **AUF1 overexpression.** See text for details.
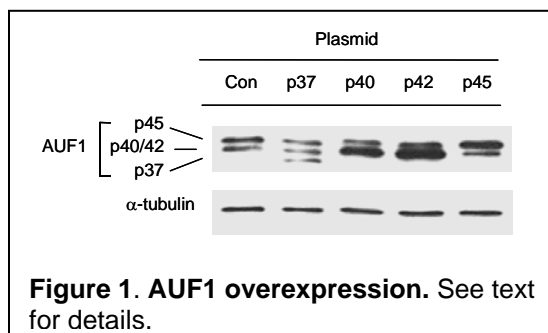
For Tasks 1-b, -c, and -d, we completed preparation of a tetracycline-regulated expression construct encoding a short hairpin RNA (shRNA) designed to knockdown expression of all four AUF1 isoforms by RNA interference (RNAi). As shown in the report for year 03, this shRNA is very effective for RNAi-induced knockdown of AUF1. We then proceeded with transfections for selection of clones. We also constructed a vector encoding a shRNA with random sequence to serve as a control (referred to as shCTL). Clones were selected, expanded, and frozen in liquid nitrogen. We froze two clones for the control shRNA (shCTL) and nine clones for the AUF1 shRNA. Our intent was for the shRNAs to be inducible by culturing cells in doxycycline (Dox). However, as we showed in last year's report,

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AUF1 knockdown occurs even in the absence of Dox for all clones tested. We estimated that AUF1 knockdown is at least 95% in all nine clones tested.

In year 03, we also demonstrated that c-*myc* mRNA is a binding target of AUF1 and that AUF1 knockdown in MCF-7 cells elevates c-Myc protein levels without effect upon mRNA abundance. This suggested to us that AUF1 controls translation, not degradation, of c-*myc* mRNA. AUF1 knockdown also increases the proliferation rate of MCF-7 cells, consistent with elevated c-Myc protein. Finally, AUF1 knockdown altered the cell-cell adhesion properties of MCF-7 cells: normally, MCF-7 cells are quite difficult to disperse, even after trypsin treatment. By contrast, cells with AUF1 knockdown are readily dispersed – even in cell culture, they do not form the epithelial "sheets" that control cells do. Importantly, all clones with AUF1 knockdown exhibited a similar defect in cell-cell adhesion.

To determine a molecular basis for the effects of AUF1 knockdown, we focused on Tasks 1-e and -f during the final year. Specifically, our goal was to identify mRNAs to which AUF1 binds so that we could examine the effects of AUF1 knockdown upon their expression using our MCF-7 cell lines. We will refer to these mRNAs as **A**UF1 **t**arget **m**RNAs, or ATMs. An earlier collaboration with Jim Malter (University of Wisconsin-Madison) led to development of a procedure to purify and identify ATMs from a complex mRNA population. We utilized mRNA prepared from peripheral blood mononuclear cells (PBMCs) (Bhattacharya et al., 1999). This earlier method purified a high background of transcripts (e.g., ribosomal protein mRNA) due to their being very abundant in PBMCs (and all cell types for that matter). ATM identification also required a cumbersome cDNA library construction protocol followed by cDNA sequencing. We have since developed modifications to increase specificity and ease of ATM identification/validation in a procedure we refer to as PACER (**P**urification and **A**mplification of **Ce**llular **R**NA). We detail the improvement, utilization, and validation of PACER below.

Figure 2. Scheme for mRNA target purification. See text for details.
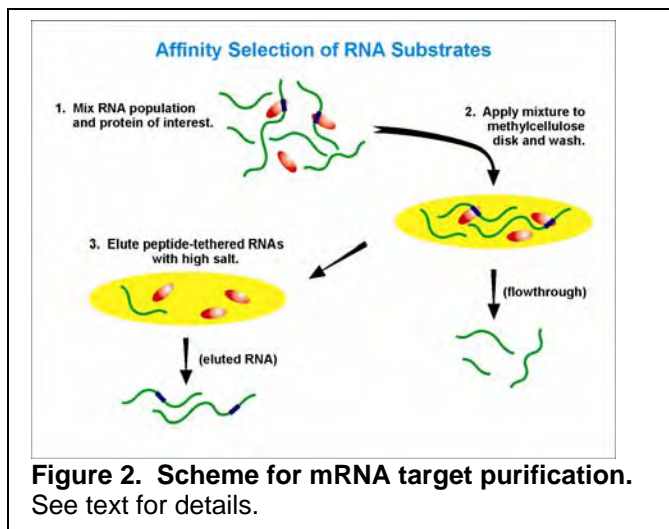
As depicted in Figure 2, PACER involves purification of ATMs by mixing a complex population of mRNA with purified, recombinant AUF1, collecting RNA:AUF1 complexes on methylcellulose discs, eluting bound RNA, reselecting eluted RNA with fresh AUF1 to increase specificity of RNA purification, again eluting reselected RNA from the methylcellulose disc, and then either amplifying the RNA by RT-PCR or using it to probe a microarray for ATM identification.

Our improvements involved altering salt concentrations during binding, washing, and elution steps and improving recovery of nanogram amounts of RNA from dilute solutions. Some initial trials of the AUF1-affinity purification strategy were performed using poly(A$^+$) mRNA. Total cellular RNA was purified using TRIzol Reagent (Invitrogen), and the poly(A$^+$) fraction was isolated using the PolyATtract kit (Promega). ^{32}P-labeled tracer RNAs were synthesized by *in vitro* transcription and included a high affinity AUF1-binding transcript (*fos* ARE, $K_d \approx 10$ nM; DeMaria and Brewer, 1996) as well as a
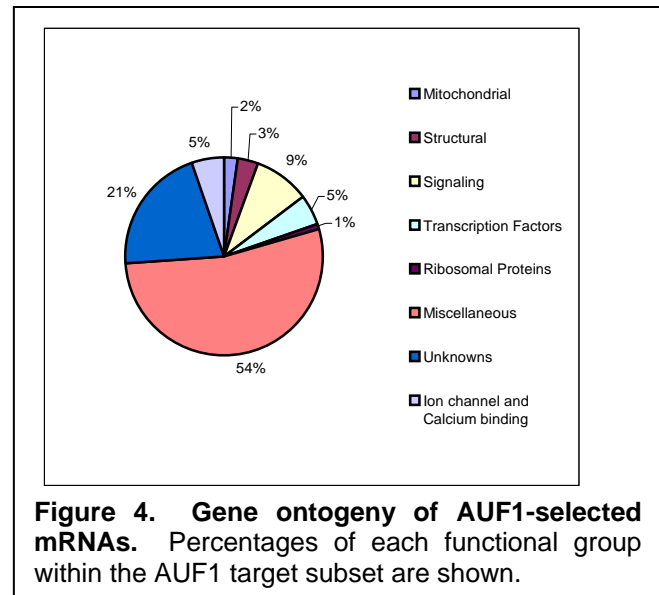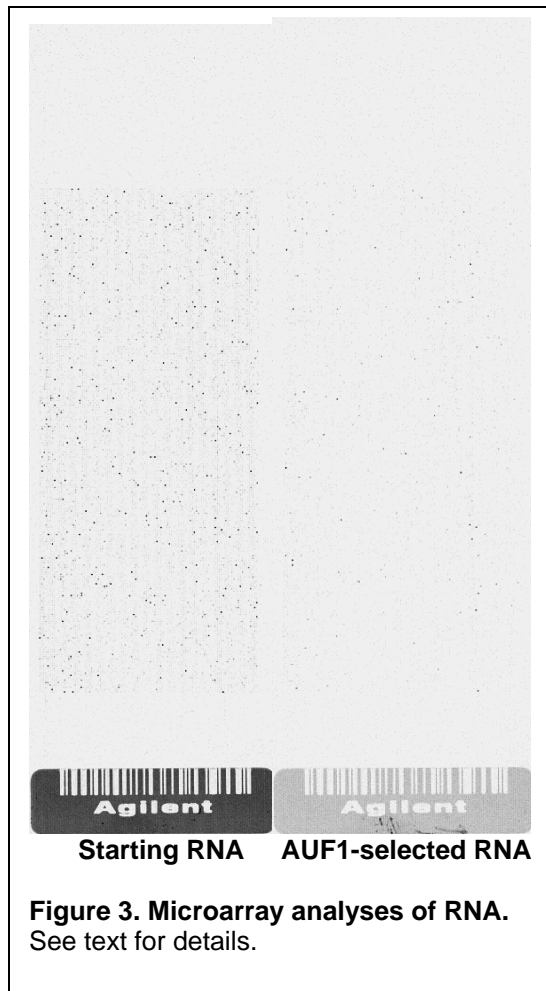
transcript which does not associate with AUF1 (ß-globin, $K_d \gg 2000$ nM). In parallel assays, 2 µg poly(A^+) mRNA was mixed with 100 fmol of either ^{32}P-*fos* ARE or ^{32}P-globin RNAs and fractionated using PACER. One micromolar of recombinant p37^{AUF1} was added to appropriately buffered RNA mixtures and incubated for 30 minutes on ice. The binding mixtures were poured onto methylcellolose discs that were prewetted with buffer. The discs were placed over a gentle vacuum and rinsed with buffer to remove any unbound RNA. Following filter washes, protein-bound RNA was recovered from the filters by increasing the salt concentration and heating. Aliquots of recovered RNA were monitored for retention of ^{32}P-labeled tracers by liquid scintillation counting. The results from a representative experiment consisting of two rounds of selection are shown below (Table 1).

Table 1: Retention of *fos* ARE and ß-globin transcripts by AUF1 affinity chromatography		
Round	*fos* ARE (fmol)	ß-globin (fmol)
0	100	100
1	34.4	0.63
2	13.7	below detection

Typically, 35-40% of the input *fos* ARE RNA was cumulatively recovered following each round, representing >50-fold retention relative to the ß-globin probe. While the ß-globin RNA remaining following the second round of selection was below the background limit for this assay, by extrapolation we predict >2500-fold enrichment of specific AUF1-binding RNAs over non-binding transcripts at this stage. This suggested that the AUF1-PACER procedure could generate a highly enriched pool of AUF1 target mRNA molecules for subsequent analyses by microarray or for RT-PCR.

Following the encouraging results of this pilot experiment, RNA was again utilized for two rounds of purification using purified AUF1, as described above. Selected RNA was then used to probe microarray chips. The Human 1A(V2) Oligo Microarray chip (Agilent), which contains over 18,000 human genes, was used to identify mRNAs interacting with AUF1. For microarray analyses, dual-color labeling was used per the manufacture's directions. Equal amounts of two RNA pools were labeled: one was starting RNA material; the other was AUF1-selected RNA. RNA samples were reverse transcribed in the presence of Cyanine dyes, one sample being labeled with Cy5 UTP and the other with Cy3 UTP. The two labeled cDNA samples was mixed, dried, and resuspended in hybridization buffer. The mixture was then incubated with the Agilent chip for 17 hours at 65°C to permit hybridization. To prevent data misinterpretation from dye bias, reciprocal labeling reactions were performed and incubated with a second chip. The arrays were scanned using a PerkinElmer Scanarray microarray scanner. Raw data were analyzed and normalized using scanarray software from PerkinElmer. As can be seen in Figure 3 (left panel), we start with a complex RNA population. However, selection with p37^{AUF1} retains a limited number of mRNAs comprising the set of AUF1 target mRNAs (Figure 3, right panel). By contrast, a control selection using BSA as protein ligand yielded little RNA compared to purifications with p37^{AUF1}.

Figure 4.   Gene ontogeny of AUF1-selected mRNAs. Percentages of each functional group within the AUF1 target subset are shown.

Starting RNA   AUF1-selected RNA

Figure 3. Microarray analyses of RNA. See text for details.

To determine preferential selection, we used the software package Limma from Bioconductor, run through the R Statistics software package. This program uses Bayesian statistics as outlined by Lönnstedt and Speed (2002). The B statistic is a penalized version of the t statistic (from student's t test), and it corrects for errors that may be incurred when sample variances are too large or small. Thus, samples with a B statistic greater than zero have a >50% chance of being selected by AUF1 (Lönnstedt and Speed, 2002).   Based upon this analysis, over 1,000 transcripts were enriched in the AUF1-selected pool. Table 2 (next page) is a list of some of the enriched mRNAs after selection with p37^{AUF1}. The B statistic has a positive correlation with enrichment after p37^{AUF1} selection. Thus, mRNAs that are most highly enriched are found at the top of the list with enrichment decreasing as the B statistic decreases. We have begun a more detailed analysis of the AUF1 targets and have been able to classify AUF1 target mRNAs into functional groups (Figure 4). Clearly, the selected pool shows that AUF1 has a diverse range of target mRNAs. Noteworthy is the presence of a significant number of ATMs encoding transcription factors.   This observation prompts consideration of the possibility that alterations in AUF1 levels could contribute significantly to substantial reprogramming of gene expression observed in breast cancer. Also noteworthy is that 21% of the selected target mRNAs encode proteins of unknown function. Finally, in our earlier study, 22% of the selected mRNAs encoded ribosomal proteins (Bhattacharya et al., 1999). Here, 1% of the PACER-selected pool encodes ribosomal proteins, the mRNAs of which are very abundant. The low representation of ribosomal protein mRNAs retained illustrates the stringency of our improved method.

Table 2: Some genes enriched by AUF1 selection		
Gene Name	Locus ID	B Statistic
Involucrin	3713	10.28631
chromosome 1 open reading frame 35	79169	10.13978
XTP3-transactivated protein B	27248	9.677383
human immunodeficiency virus type I enhancer binding protein 2	3097	8.147781
v-akt murine thymoma viral oncogene homolog 3 (protein kinase B	10000	7.899202
phorbol-12-myristate-13-acetate-induced protein 1	5366	7.6372
mesenchymal stem cell protein DSC43	51333	6.751877
prion protein 2 (dublet)	23627	6.73883
superoxide dismutase 2	6648	6.329608
Defensin	140596	6.207512
lymphocyte antigen 6 complex	4062	5.7257
CTP synthase	1503	4.989264
hypothetical protein FLJ32569	148811	4.944573
cyclin-dependent kinase (CDC2-like) 10	8558	4.847751
zinc finger protein 618	114991	4.109863
pre-B-cell leukemia transcription factor 3	5090	3.975743
Prolactin receptor	5618	3.628976
MAX dimerization protein 5	23269	3.382137
hydroxysteroid (17-beta) dehydrogenase 2	3294	3.262046
PR domain containing 14	63978	3.09105
Sidekick homolog 1 (chicken)	221935	2.992786
protein tyrosine phosphatase	5770	2.838281
Zonadhesin	7455	2.767778
homeodomain interacting protein kinase 2	28996	2.755512
solute carrier family 1 (neutral amino acid transporter), member 5	6510	2.699796
DnaJ (Hsp40) homolog, subfamily B, member 12	54788	2.686434
solute carrier family 38	10991	2.615986
ARF protein	51326	2.487991
solute carrier family 22 member 7 isoform a	10864	2.202301
propionyl Coenzyme A carboxylase	5095	2.199478
hypothetical protein FLJ22955	79877	1.986597
glutamate receptor	2905	1.917653
RAS p21 protein activator 3	22821	1.852522
hypothetical protein FLJ13639	79758	1.847005
meteorin, glial cell differentiation regulator-like	284207	1.70377
ankyrin repeat and SOCS box-containing 1	51665	1.373251
high density lipoprotein-binding protein	338328	1.2748
solute carrier family 2 (facilitated glucose/fructose transporter)	6518	1.105148
hypothetical protein FLJ21628	80108	1.089588
Tubulin	81027	1.085537
major histocompatibility complex, class II, DR alpha precursor	3122	0.820339
chorionic gonadotropin	93659	0.780722
Putative secreted protein ZSIG11	51368	0.764933
formin homology 2 domain containing 1	29109	0.733375

We also analyzed the 3'-UTRs of the first 80 selected transcripts with the highest *B* statistic. Of these transcripts, approximately 50% have AREs (or ARE-like sequences) within their 3'-UTRs. Representative ARE sequences can be found in Table 3. These observations also raise the likelihood that the target binding site of p37^{AUF1} in RNA may well extend beyond AREs. Future experiments will bring us closer to elucidating the effects of AUF1 levels on the expression of these genes, both ARE and non-ARE alike.

Table 3: Representative A+U-rich sequences from selected mRNAs

CTP synthase
   aaattatgtt tttattaaga ttattttatt
Unknown

   tgcctattta **taattaaagt atttttcttt agtttgaaat gtgtattaaa gttacatttt tgagttacaa gagtcttata actacttgaa tttttagtta aaatgtctta atgtaggttg tagtcacttt agatggaaaa ttacctcaca tctgttttct tcagtattac ttaagattgt tt**atttagtg **gtagagagat ttttttttc agcctagagg cagctatttt accatctggt **atttat**ggtc taatttgt**at **tt**aaacatat
Pre-B-cell leukemia transcription factor 3
   aagatttaac **attgttgaca gtcctgtagc tattttatca ta**atttatta **tcaatatttt**
Protein tyrosine phosphatase, non-receptor type 1
   att **tatttaaaca **attttttccc caaaggcatc catagtgcac tagcattttc ttgaaccaat aatgtattaa aattttttga

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To validate the enrichment and purification of specific transcripts as indicated by microarray analyses, we performed qRT-PCR. We chose transcripts that were representative of differential *B* statistics and designed primers for the given genes.   Table 4 (next page) contains the genes we examined and their respective *B* statistics. We then performed qRT-PCR with two RNA samples: one was the AUF1-selected RNAs; the other was the starting RNA population. By comparing ΔC_t values for a given transcript in the two samples, we expected to see a trend whereby *B* statistics would be directly proportional to the magnitude of the enrichment, as noted above. For example, a gene with a large, positive-valued *B* statistic would have a large enrichment factor. By contrast, a gene with a negative-valued *B* statistic would have a low (or negative) enrichment value (i.e., it would be less abundant in the AUF1-selected RNA population compared to the starting RNA population). This latter population would likely represent transcripts obtained by PACER, not based upon their high affinity interaction with AUF1, but rather their relatively high abundance. Figure 5 (next page) demonstrates that transcripts with a high *B* statistic were, in fact, highly enriched (e.g., superoxide dismutase 2). Transcripts with a negative *B* statistic (e.g., chromobox homolog) were selected against and consequently have a negative enrichment value.   Thus, the qRT-PCR experiment has, so far, validated the microarray analyses of the AUF1-selected RNA population. We conclude that our microarray approach is efficacious for identifying the RNA target population of AUF1 and quantitatively assessing the enrichment factor following purification by PACER. This approach should also be useful for any RNA-binding protein of interest. We are also poised to complete the mapping of ATMs using the other AUF1 isoforms, as well as continuing the validation of microarray data by qRT-PCR. This latter goal should be greatly facilitated by the commercial availability of qPCR primer banks for thousands of genes (e.g., from SuperArray Bioscience).

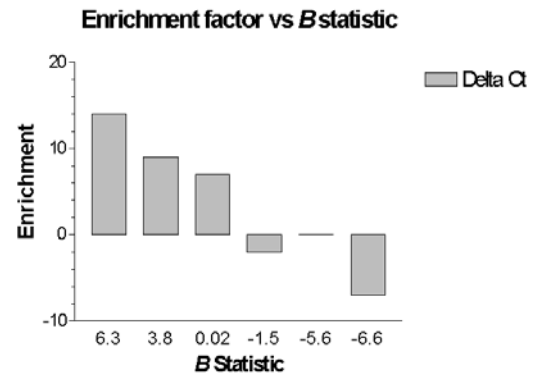| Table 4: Genes for RT-PCR and their *B* statistics	
Genes chosen for analysis	*B* statistic
Enriched genes	
Superoxide dismutase 2	6.33
sidekick homolog 1 (chicken)	2.99
cytochrome P450, family 2, subfamily J, polypeptide 2	0.02
Non-enriched genes	
chromobox homolog	-1.52
phosphoprotein associated with glycosphingolipid-enriched membranes	-5.58
FLJ10134	-6.38

Figure 5. qRT-PCR validation of PACER. See text for details.

KEY RESEARCH ACCOMPLISHMENTS (years 01-04)

- Completion of vectors for overexpression or knockdown of AUF1 and HuR.
- Creation of cloned cell lines of MCF-7 with >95% knockdown of AUF1 expression compared to cell lines expressing a control shRNA.
- Identification of c-*myc* mRNA as an AUF1 target mRNA in cells.
- Discovered that AUF1 is a novel translational repressor of c-*myc* mRNA.
- Knockdown of AUF1 accelerates proliferation of MCF-7 cells and disrupts their cell-cell adhesion properties.
- Designed a genetic system for complementation of individual AUF1 isoforms in knockdown cells to assess the effects on cell proliferation and cell-cell adhesion in culture and in animals.
- Developed an improved method to purify AUF1 target mRNAs from a complex mRNA population.
- Identified subset of mRNAs bound by p37 AUF1 isoform.
- Validated the purification procedure using quantitative RT-PCR.

REPORTABLE OUTCOMES

We have in hand all the tools that will allow us to assess the biological effects of reengineering expression of the key ARE-binding protein AUF1 in human breast carcinoma cells. We have also made two seminal observations: AUF1 is a translational repressor of c-*myc* mRNA in breast carcinoma; and AUF1 knockdown blocks the cell's ability to form stable cell-cell contacts. These observations, as such, are not yet reportable outcomes, as we need to establish the responsible molecular mechanisms. As such, we initiated AUF1 mRNA target identification by PACER and microarray analyses. These data should help us to identify gene subsets that contribute to AUF1 function, in particular how AUF1 affects cell-cell adhesion.

CONCLUSIONS

Our central hypothesis has been that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth, and that HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach was to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Our work had two phases: (I) to examine the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (II) to assess the roles of AUF1 and HuR in cellular proliferation and tumorigenesis *in vivo*. Over the past four years, we completed expression vector constructions, transfections, and clone selections germane to Phase I. Stable overexpression or knockdown of HuR was problematic. Knockdown of AUF1 was achieved, however, and led to two very important observations: (i) AUF1 acts to repress translation of c-*myc* mRNA in breast carcinoma cells to limit protein synthesis. As such, AUF1 does operate as a tumor suppressor, at least in breast cancer. (ii) Knockdown of AUF1 disrupts cell-cell adhesive properties of breast epithelial cells. We predict that these cells will exhibit a highly aggressive, metastatic phenotype. We developed a means to complement expression of any AUF1 isoform within a background of knocked down, endogenous AUF1 expression. This novel and powerful genetic system will be a gold mine for dissecting new mechanisms and pathways by which breast carcinoma cells become highly metastatic during advanced breast cancer. Finally, to understand the biology of AUF1, we used an improved method to identify mRNA subsets to which it binds to effect its functions.

Analyses of the mountains of microarray data obtained are far from complete; this task will require many, many months.   If possible, we also hope to begin the *in vivo* studies with nude mice in the near future.

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APPENDICES

None

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